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Ontogeny and social context regulate the circadian activity patterns of Lake Malawi cichlids

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16 **Abstract**

17 Activity patterns tend to be highly stereotyped and critical for executing many different behaviors
18 including foraging, social interactions and predator avoidance. Differences in the circadian timing
19 of locomotor activity and rest periods can facilitate habitat partitioning and the exploitation of novel
20 niches. As a consequence, closely related species often display highly divergent activity patterns,
21 raising the possibility that a shift from diurnal to nocturnal behavior, or vice versa, can occur
22 rapidly. In Africa's Lake Malawi alone, there are over 500 species of cichlids, which inhabit diverse
23 environments and exhibit extensive phenotypic variation. We have previously identified a
24 substantial range in activity patterns across adult Lake Malawi cichlid species, from strongly
25 diurnal to strongly nocturnal. In many species, including fishes, ecological pressures differ
26 dramatically across life-history stages, raising the possibility that activity patterns may change
27 over ontogeny. To determine if rest-activity patterns change across life stages we compared the
28 locomotor patterns of six Lake Malawi cichlid species. While total rest and activity did not change
29 between early juvenile and adult stages, rest-activity patterns did, with juveniles displaying distinct
30 activity rhythms that are more robust than adults. One distinct difference between juveniles and
31 adults is the emergence of complex social behavior. To determine whether social context is
32 required for activity rhythms, we next measured locomotor behavior in group housed adult fish.
33 We found that when normal social interactions were allowed, locomotor activity patterns were
34 restored, supporting the notion that social interactions promote circadian regulation of activity in
35 adult fish. These findings reveal a previously unidentified link between developmental stage and
36 social interactions in the circadian timing of cichlid activity.
37

38 Introduction

39

40 Animals display remarkable diversity in rest-activity patterns^{1,2}. The timing of rest and activity can
41 differ dramatically between closely related species, or even between populations of the same
42 species, raising the possibility that it can be adaptive and subject to selection^{3,4}. Indeed, circadian
43 regulation of locomotor activity is strongly associated with many factors critical in determining
44 organismal fitness, including foraging strategy, social behavior, and predator avoidance^{5,6}.
45 Further, rest-activity patterns are acutely regulated by environmental factors and life-history traits
46 that include food availability, social interactions, and age^{7,8}. Defining how complex environmental
47 interactions regulate activity patterns is therefore critical to understanding behavioral adaptation
48 and evolution.

49

50 There are over 35,000 teleost species, adapted to diverse habitats, and representing ~50% of
51 vertebrate diversity⁹. Many teleosts display robust diurnal locomotor rhythms including the
52 goldfish (*Carassius Auratus*), river-dwelling populations of the Mexican tetra (*Astyanax*
53 *mexicanus*), and the zebrafish (*Danio rerio*)¹⁰⁻¹³. Examples of nocturnal teleosts have also been
54 identified including the plainfin midshipman, the Senegalese sole, and the doctor fish, *Tinca*
55 *tinca*¹⁴. Other species such as cavefish morphs of the Mexican tetra, *A. mexicanus*, and the
56 Somalian cavefish, *Phreatichthys andruzzii*, and cave dwelling populations of *A. mexicanus* have
57 largely lost light-driven circadian regulation of behavior¹⁵⁻¹⁸. Despite the characterization of
58 species from disparate lineages/populations, few studies have examined how developmental
59 stage, or divergent ecological contexts regulate sleep among closely related species.
60 Understanding the variability of rest-activity patterns over phylogeny, ecology and ontogeny,
61 therefore represents a vital step toward identifying conserved genetic and evolutionary features
62 that may influence regulation of activity throughout vertebrates.

63

64 Lake Malawi cichlids exhibit unparalleled diversity in morphology and behavior among
65 vertebrates¹⁹⁻²¹. Observations at night suggest that adult cichlids may be diurnal as an evolved
66 strategy for predator avoidance, at least for species occupying near-shore habitats²². Specifically,
67 Lake Malawi is home to endemic non-cichlid predators, including the Cornish jack *Mormyrops*
68 *anguilloides*, which feeds in packs at night using weak electrical pulses thought to be undetectable
69 by cichlids²³. We previously analyzed the activity patterns of 11 species of cichlids, from diverse
70 habitats and distinct genetic lineages and found significant variability ranging from highly

71 nocturnal to diurnal, with many species exhibiting no differences in day or night activity²⁴. In a
72 single identified nocturnal species, *Tropheops*. sp. “red cheek,” the pattern held at two life-history
73 stages (i.e., late juvenile vs. mature adult), and under different abiotic conditions (i.e., presence
74 vs. absence of shelter)²⁴. However, for many species there was no preference for light or dark
75 activity, raising the possibility that activity patterns are either context dependent or absent in some
76 species²⁴.

77
78 Here, we focused on the activity patterns of Lake Malawi cichlids that seem to lack activity patterns
79 during adulthood. All Lake Malawi cichlids are maternal mouth brooders, and we found that as
80 newly emerged fry (i.e., early juvenile stage) all six species exhibited robust rest-activity patterns.
81 Thus, we demonstrate ontogenic regulation of activity patterns across diverse cichlids.
82 Furthermore, in a subset of species, we find that activity patterns of adults were largely restored
83 under social housing conditions. Together, these studies demonstrate the complexity and context
84 dependency of the circadian regulation of rest-activity in Lake Malawi cichlids.

85

86

87 **Results**

88 ***Total activity is similar between juvenile and adult cichlids***

89 We examined six Lake Malawi cichlids from three distinct lineages (Fig 1A). The *mbuna* lineage
90 is phylogenetically monophyletic, with species generally inhabiting the near-shore rocky habitat.
91 Three *mbuna* species were utilized here: *Labidochromis caeruleus* (Lundo Island),
92 *Melanochromis heterochromis* (Mumbo Island), *Tropheops kumwera* (Kanchedza Island). All
93 three are territorial and sexually dimorphic as adults. *Labidochromis* species are generally
94 omnivorous, consuming both benthic invertebrates, algae, and plankton. *M. heterochromis* has a
95 similarly omnivorous diet. *T. kumwera* feeds primarily on benthic algae^{25,26}. *Astatotilapia calliptera*
96 is sister to the *mbuna*, and has been called the “most generalized species in the lake”²⁶. It’s
97 generalist designation refers to both diet and habitat, as it one of few Lake Malawi species that
98 inhabits the surrounding river systems. Both *Aulonocara stuartgranti* and *Nimbochromis venustus*
99 are non-*mbuna* species, which is a polyphyletic group of cichlids that generally occupy deeper,
100 open-water, and/or sandy habitats. *A. stuartgranti* feeds on benthic invertebrate that it locates
101 using an enlarged lateral line system, whereas *N. venustus* is an open-water piscivore. All fish
102 were obtained from the aquarium trade. While generation from the wild cannot be verified for
103 these animals, breeding populations are maintained for species, and often to specific
104 locations/populations in the lake, with new individuals introduced to the breeding pool

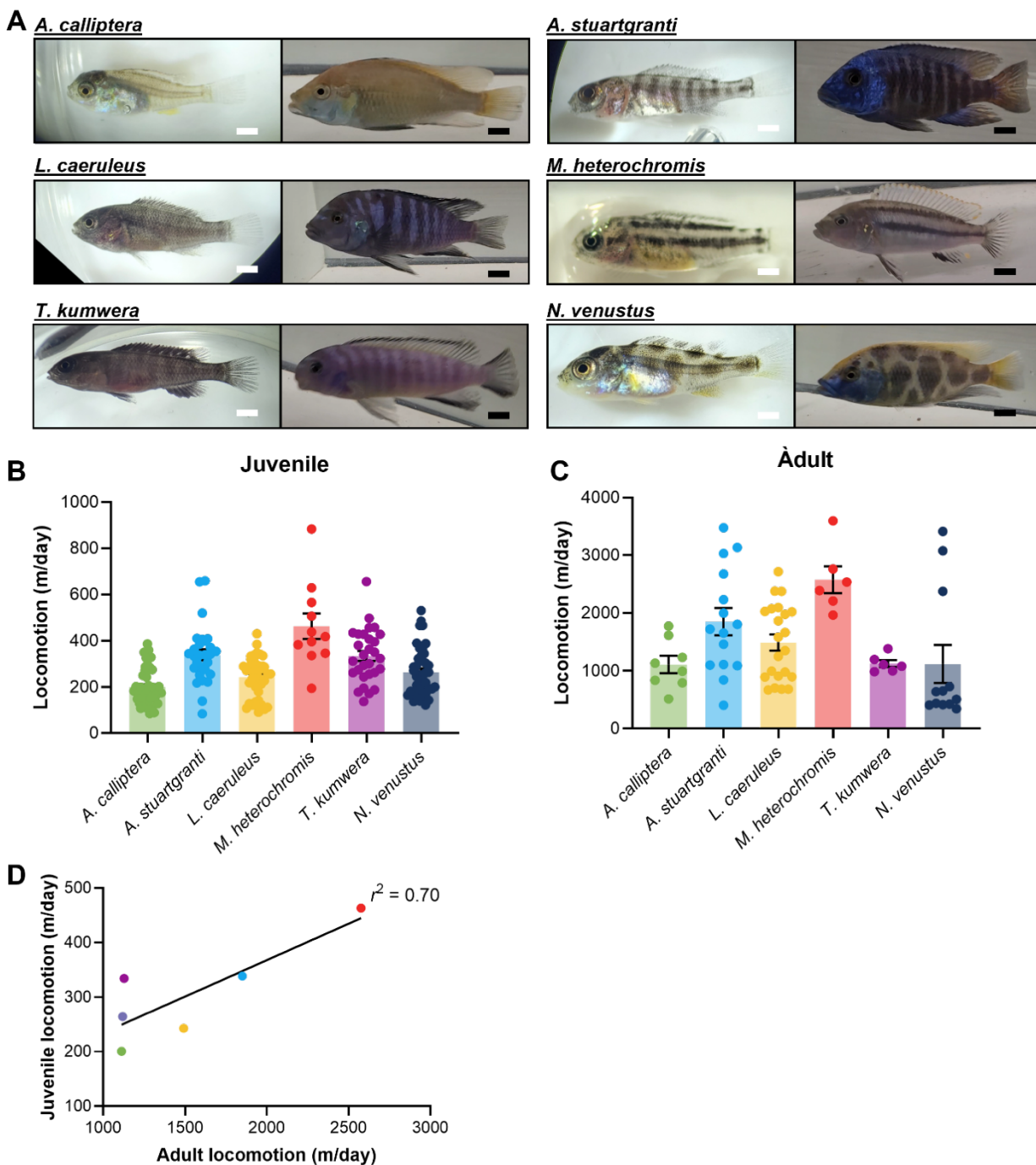


Figure 1. Variation in cichlid activity levels is conserved throughout development. **A.** Images of early juvenile (left) and adult (right) cichlid species used in the present study. Juveniles were tested and photographed between 3-4 weeks post-fertilization, shortly after the depletion of the yolk sac and at the earliest free-swimming stage. Adults were tested and photographed at full maturity, after the development of nuptial colors. White Scale Bar (Juveniles) = 1 mm. Black Scale Bar (Adults) = 1 cm. **B.** Total locomotion of juvenile cichlids over a 24 hour period. There is significant variation in total locomotion in juvenile cichlids (ANOVA: $F_{5, 190} = 15.99, p < 0.0001$). **C.** Total locomotion of adult cichlids over a 24 hour period. There is significant variation in total locomotion in adult cichlids (ANOVA: $F_{5, 67} = 6.42, p < 0.0001$). **D.** Correlation between 24-hour locomotion in juveniles and adults. There is a significant correlation between juvenile and adult locomotion ($r^2 = 0.70, p = 0.0388$)

106 to maintain genetic health.

107

108 To measure activity in cichlids we used systems similar to those established in other fish species
109 including zebrafish and *A. mexicanus*²⁷⁻²⁹. For juveniles, we used 6-well tissue culture plates,
110 whereas adults were filmed in 2.5-gallon glass aquaria. Following 24 hours of acclimation,
111 individually housed fish were filmed for a 24 hour period under standard 14 hr light: 10 hr dark
112 conditions. We noted significant variation in total activity with and between species of juvenile and
113 adults (Fig. 1B, C). For example, *A. calliptera* was the least active in both juveniles and adults,
114 while *M. heterochromis* had the highest activity levels at both stages, suggesting total activity is
115 conserved across developmental stages (Fig 1B,C). To directly test this notion, we examined the
116 relationship between total activity in juveniles and adults. A regression revealed a strong positive
117 relationship ($r^2=0.70$, $p=.038$) across species between juvenile adult locomotor activity (Fig 1D).
118 Together, these data reveal significant inter-species variation that remains constant across the
119 life cycle.

120

121 ***Juvenile cichlids exhibit robust activity patterns compared to adults***

122 We next sought to understand whether rest-activity patterns changed between life-history stages.
123 In juveniles, we identified robust activity patterns across all six species tested (Fig 2A,C). In total
124 there were four diurnal species (*A. calliptera*, *L. caeruleus*, *M. heterochromis*, and *T. kumwera*)
125 and two nocturnal species (*A. stuartgranti* and *N. venustus*). Conversely, there were no significant
126 differences between day and nighttime activity across all six species when tested during
127 adulthood (Fig 2B,D). These results were confirmed when we computed a diurnality index, which
128 compares daytime to nighttime activity in individual fish (Fig 2E)²⁴. There was significantly more
129 time-of-day activity preference in five of the six species tested (Fig 2E). Thus, activity patterns
130 appear to be more robust in early juvenile cichlids compared to adult fish under individually
131 housed conditions.

132

133 Across taxa, the timing of behavioral quiescence or rest are modulated by the circadian clock and
134 linked to daily activity patterns^{3,4}. To examine whether the timing of rest differs across life stages,
135 we therefore compared rest periods of one minute or longer, a timeframe of inactivity that is used
136 to define sleep in related species³⁰. There were robust differences in total rest across juvenile and
137 adult species (Fig 3A-D). Similar to the analysis of activity, there was consistency in the duration
138 of rest between juveniles and adults. For example, *M. heterochromis* had the lowest levels of rest
139 in juveniles and adults, whereas *A. calliptera* and *N. venustus* had the highest level at both

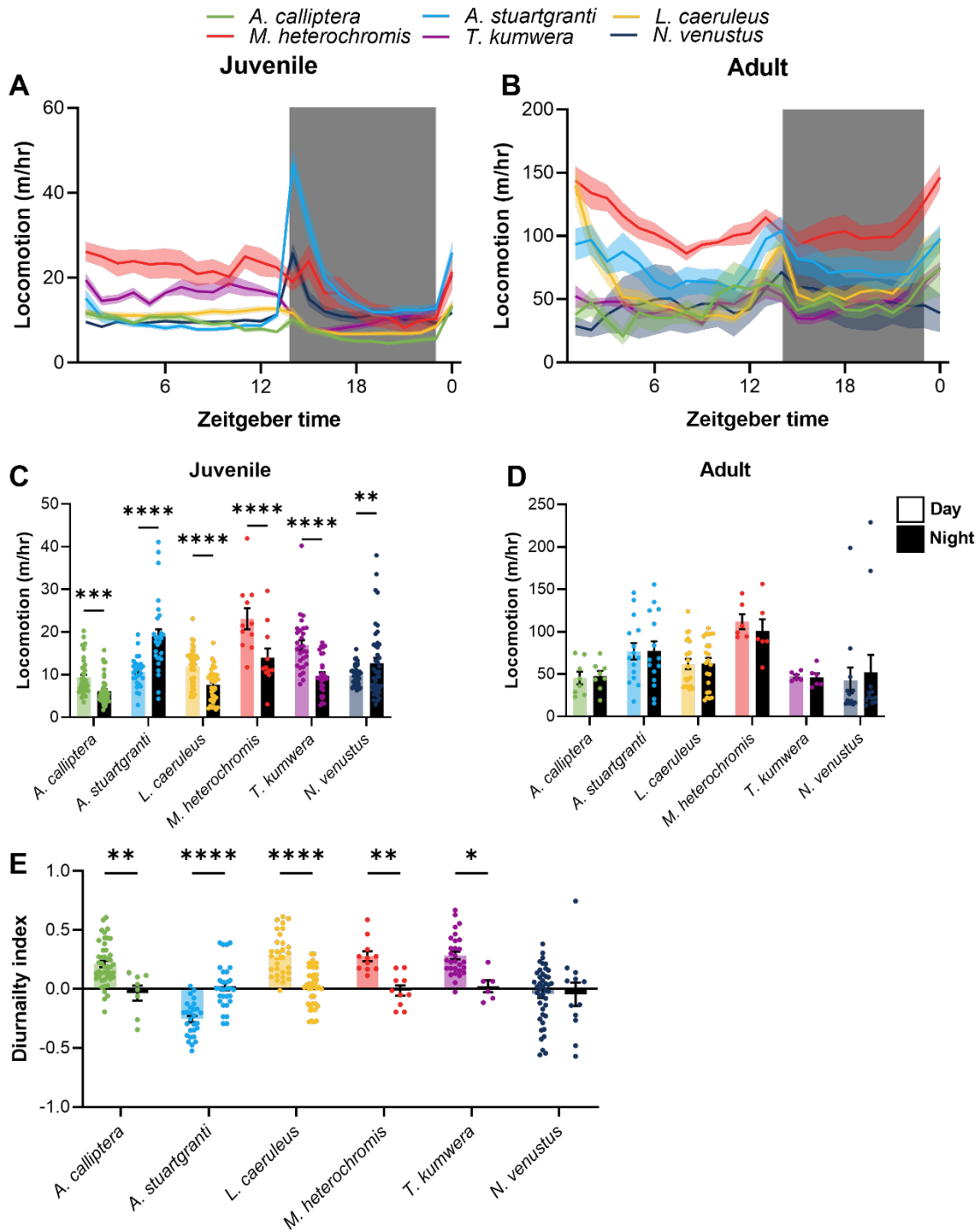


Figure 2. Ontogeny of behavioral rhythms in locomotion. **A.** Locomotion profiles of juvenile cichlids over 24 hours. Shaded area indicates dark period. **B.** Locomotion profiles of adult cichlids over 24 hours. Shaded area indicates dark period. **C.** Average hourly locomotion of juvenile cichlids during the day and night. Juvenile cichlids exhibit diurnal (*A. calliptera*, *L. caeruleus*, *M. heterochromis*, *T. kumwera*) or nocturnal (*A. stuartgranti*, *N. venustus*) activity patterns (two-way ANOVA: $F_{5,190} = 36.54$, $p < 0.0001$). **D.** Average hourly locomotion of juvenile cichlids during the day and night. Adult cichlids lack behavioral rhythms when tested in isolation. **E.** Strength of behavioral rhythms in juvenile and adult cichlids. 1 indicates total diurnality, -1 indicates total nocturnality. Behavioral rhythms are present in juveniles but not adults (two-way ANOVA: $F_{5,280} = 16.20$, $p < 0.0001$).

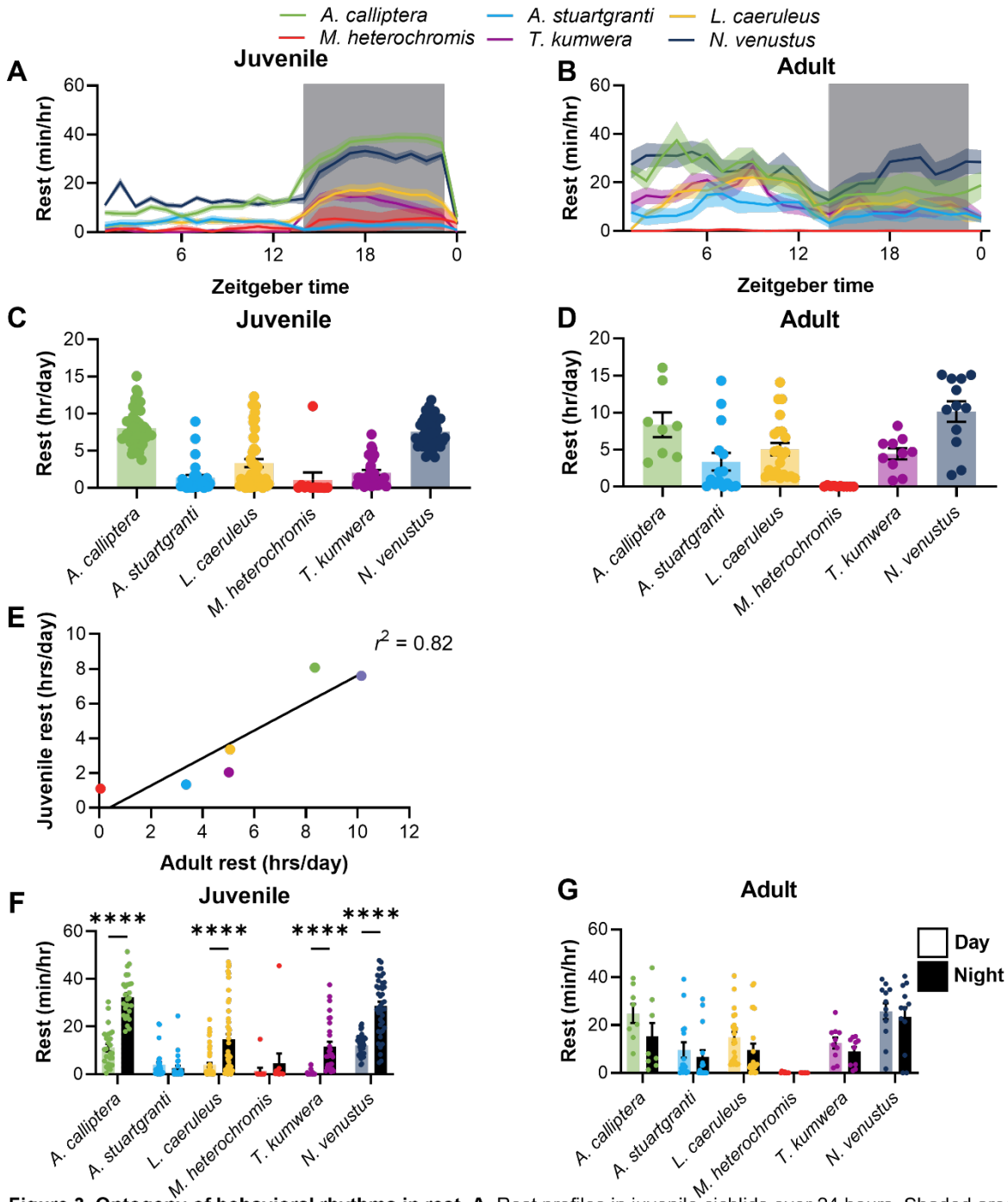


Figure 3. Ontogeny of behavioral rhythms in rest. **A.** Rest profiles in juvenile cichlids over 24 hours. Shaded area indicates dark period. **B.** Rest profiles in adult cichlids over 24 hours. Shaded area indicates dark period. **C.** Total rest amounts over 24 hours in juvenile cichlids. There is significant variation in total rest amount in juvenile cichlids. (ANOVA: $F_{5, 190} = 42.4, p < 0.0001$). **D.** Total rest amounts over 24 hours in adult cichlids. There is significant variation in total rest amount in adult cichlids (ANOVA: $F_{5, 67} = 8.792, p < 0.0001$). **E.** Correlation between juvenile and adult rest amounts. There is significant correlation between juvenile and adult rest ($r^2 = 0.82, p < 0.0128$). **F.** Average hourly rest amount of juvenile cichlids during the day and night. Juvenile cichlids exhibit diurnal (*A. calliptera*, *L. caeruleus*, *T. kumwera*, *N. venustus*) or nocturnal (*A. stuartgranti*) rest patterns (two-way ANOVA: $F_{5, 358} = 10.95, p < 0.0001$). **G.** Average hourly rest amount of adult cichlids lack behavioral rhythms in rest amount.

142 developmental stages. Indeed, a regression analysis revealed a strong relationship ($r^2=0.82$,
143 $p=0.012$) between juvenile and adult rest duration (Fig 3E). Therefore, the duration of total rest is
144 maintained throughout development across multiple species of cichlids.

145
146 We next compared the amount of rest over the light and dark periods. Juvenile *A. calliptera*, *L.*
147 *caeruleus*, and *T. kumwera* displayed robust patterns in rest regulation with increased nighttime
148 rest, while the fourth diurnal species *M. heterochromis* trended in the same direction (Fig 3F),
149 suggesting diurnality is associated with periods of nighttime rest in juvenile cichlids. Conversely,
150 there were no differences in the timing of rest for *A. stuartgranti*, suggesting that nocturnality in
151 this species is driven by swimming velocity during wakefulness, rather than the timing of rest (Fig
152 3F). Finally, in *N. venustus*, which is nocturnal at the juvenile stage, there is robust consolidation
153 of rest during the night (Fig 3F). For all species tested there was little difference between daytime
154 and nighttime rest in adults (Fig 3G). To understand this trait, we calculated a crepuscularity index,
155 which measures the ratio of activity immediately after the light transitions relative to the rest of the
156 day. We saw notable variation across species in this measurement, with *N. venustus* and *A.*
157 *stuartgranti* displaying the highest degree of crepuscularity, increasing activity levels by up to two
158 fold in the hours following light transitions; other species showed little to no crepuscular
159 tendencies (Fig S1A). Similar to day-night behavioral rhythms, crepuscular behavior was largely
160 lost in adulthood (Fig S1B).

161
162 There were no day-night differences in rest across all six populations of adult cichlids, again
163 confirming that the timing of rest-activity is more robust in juveniles than adult fish (Fig 3G).
164 Together, these findings support the notion that rest-activity bouts are more readily consolidated
165 in juvenile fish and reveal separate mechanisms for the emergence of diurnal, nocturnal, and
166 crepuscular activity patterns.

167 168 **Social housing can restore diurnal activity in adults**

169 Many fish species, including cichlids, are highly social, with animals displaying behavioral
170 differences under solitary and group housing^{31–35}. Except for a small number of studies, nearly
171 all analysis of rest-activity patterns in fishes have used individually housed animals, including
172 cichlids. Social interactions are weaker in juvenile fish, compared to adults, raising the possibility
173 that the lack of rest-activity patterns in adults is an artifact of solitary housing conditions. To
174 examine the effects of social housing on rest-activity patterns we measure activity patterns in
175 group housed cichlids comprised of two adult males and four adult females (Fig 4A). All fish were

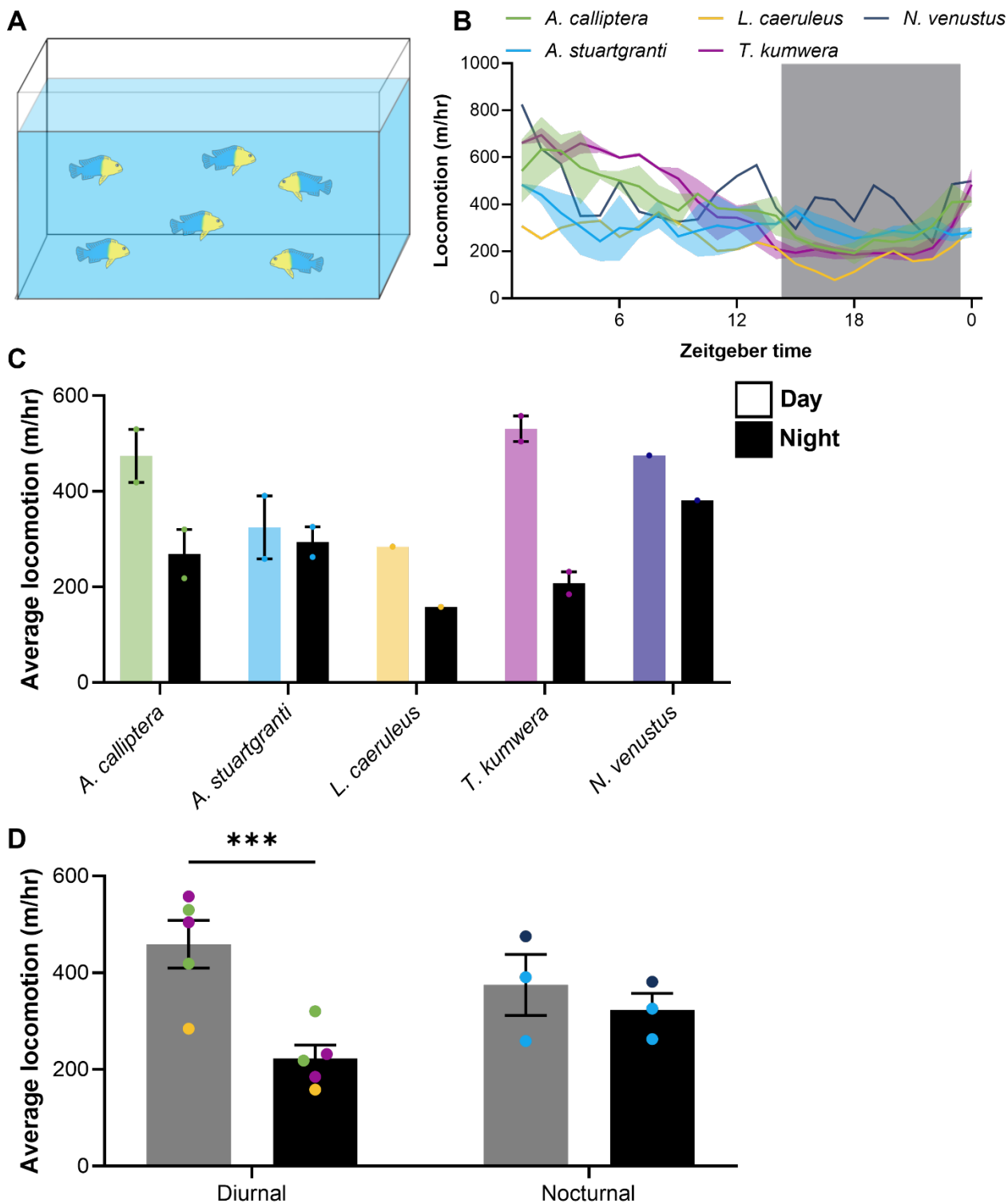


Figure 4. Social context restores rhythms in diurnal cichlids. **A.** Schematic of test setup. Adult cichlids are transferred to a 10-gallon testing aquarium, acclimated overnight, and then recorded for 24 hours. **B.** Average locomotion of cichlid groups over 24 hours. Shaded area indicates dark period. **C.** Average hourly locomotion of group housed cichlids, during the day and night. **C.** Average hourly locomotion of group housed cichlids during the day and night, grouped into diurnal and nocturnal species. Group housing restores rhythmic behavior in diurnal, but not nocturnal species (two-way ANOVA: $F_{1,6} = 11.23$ $p = 0.0154$). Colors of data points indicate species.

177 transferred into testing tanks and given 24 hours to acclimate and establish stable social
178 interactions. We tested four different cichlid species under group-housed conditions for 24 hours.
179 While we were unable to track individual fish reliably over the 24 hour period, we used average
180 activity of the group. Three of these species tested (*A. calliptera*, *L. caeruleus*, and *T. kumwera*)
181 trended towards recovery of diurnal behavior (Fig 4C). There was little difference in the species
182 *A. stuartgranti* or *N. venustus*, which are nocturnal as juveniles. To examine the broader trends
183 of sleep in group housed fish we combined analysis of nocturnal and diurnal species. Social
184 housing induced robust diurnality in species that are diurnal as juveniles, while there was no
185 differences between day and night activity in the two nocturnal species (Fig 4D). These findings
186 suggest diurnal juveniles species likely maintain activity patterns in adulthood, but the fish only
187 display these behaviors under group-housed conditions.

188
189 To examine the behavior of individual fish in more detail, we manually annotated behavior for a
190 single species over the full 24 hour testing period. We chose to examine *L. caeruleus* because of
191 their robust diurnal behavior as group-housed adults and juveniles. Fish behavior was manually
192 analyzed in Behavioral Observation Research Interactive Software (BORIS) for active swimming
193 over the 24 hour cycle³⁶. We developed an ethogram across all six fish with rest-activity timing
194 (Fig 5A). We observed robust diurnal activity across all male and female individuals (Fig 5B,C).
195 Further, the duration of rest bouts was greater during the night period (Fig 5D,E). These findings
196 confirm that diurnality is not sex-specific, and generalizable across the dominance structure.

197

198

199 **Discussion**

200 ***The importance of context in behavioral studies***

201 The genetic, morphological, and behavioral diversity of African cichlids provides an exceptional
202 model for understanding trait evolution¹⁹. In spite of myriad comparative studies in this system,
203 very few have focused on rest-activity patterns. Here, we characterize the behavior of six species
204 of Lake Malawi cichlids at multiple developmental stages, highlighting a role for ontogeny and
205 social context in the regulation of activity. Previously, we characterized rest-activity patterns in 11
206 species of Lake Malawi cichlids at the late juvenile, or sub-adult stage, identifying 7 species
207 without activity preferences, as well as two diurnal species and one nocturnal species²⁴. The lack
208 of activity preferences across the majority of species was surprising given the ubiquity of circadian
209 rhythms and the robust diurnal or nocturnal behaviors across many species of animals that have
210 been studied to date. Here, we examined species that also seemed to lack robust activity patterns
211 as adults, but showed that these behaviors are present when animals were examined at an early

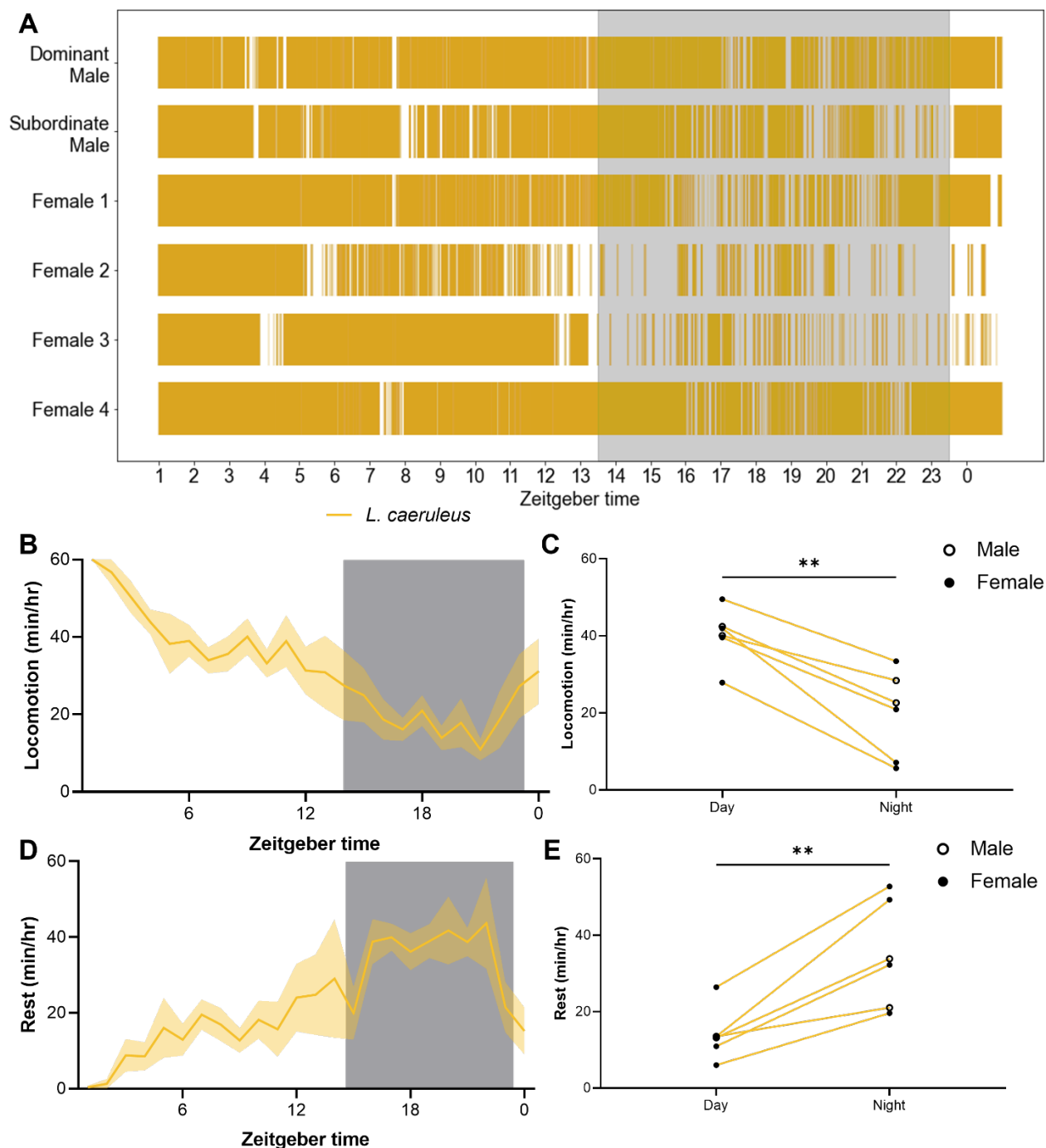


Figure 5. Individual variation in behavioral rhythms in a social context. **A.** Ethogram of active swimming behavior of *L. caeruleus* over 24 hours, in a social context. Colored regions indicate periods of active swimming. Shaded area indicates dark period. **B.** Profile of locomotion in group housed *L. caeruleus*, quantified as minutes of active swimming per hour. Shaded area indicates dark period. **C.** Average hourly locomotor activity of group housed *L. caeruleus* during the day and night. Group housed *L. caeruleus* are significantly more active during the day (Paired t-test: $t_5 = 6.413$, $p = 0.0014$). **D.** Rest profile of group housed *L. caeruleus* over 24 hours. Shaded area indicates dark period. **E.** Average hourly rest of group housed *L. caeruleus* during the day and night. Group housed *L. caeruleus* consolidate rest during the dark period (Paired t-test: $t_5 = 5.198$, $p = 0.0035$).

213 juvenile stage or within a social context. Of note, *T. kumwera* showed diurnal activity patterns in
214 our previous study, when tested at the sub-adult stage. Here, we found that this behavioral rhythm
215 is present from the earliest developmental timepoints, but is lost by the onset of sexual maturity.
216 However, placing the adult fish in a social context restored diurnal rhythmicity, underscoring the
217 importance of a social context for maintenance of behavioral rhythms in this species. These
218 findings highlight the complexity of animal behaviors, and suggest that many of the species
219 previously examined may possess innate activity rhythms when examined in a different context.

220

221 The role of circadian and sleep regulators across development remains poorly understood. For
222 example, in humans sleep varies significantly across the life cycle but little is known about the
223 factors that regulate pediatric sleep³⁷. In fruit flies, genes regulating sleep and activity in larvae
224 differ from those regulating sleep in adult flies suggesting that the genetic basis of the
225 consolidation of activity patterns is specific to developmental stages^{38,39}. Here we find that total
226 locomotor activity and duration of rest appear to be robust to life-history stage, whereas the timing
227 of rest-activity patterns is dependent upon ontogeny, raising the possibility that there are genetic
228 and contextual differences regulating the timing, but not the amount of activity. The cichlid species
229 examined here reach sexual maturity at ~6 months. This stage is associated by an increase in
230 sex-specific hormones that is associated with aggression, territoriality, mating behaviors, and
231 dominance hierarchies⁴⁰. The gain of these behaviors seems to be associated with the loss of
232 activity pattern robustness, suggesting that natural social conditions are required for the
233 expression of diurnal or nocturnal rhythms. Whether the lack of rhythms in individually housed
234 adults is due to the lack of a necessary environmental cue, or represents a secondary stress
235 response remains unclear. Taken together, our data suggests that understanding the rest-activity
236 patterns in adults will require further testing in diverse ethological contexts.

237

238 It is notable that total rest-activity appears to be robust to life-history stage, which suggests that it
239 is more hard-wired compared to rest-activity patterns. If true this would suggest greater potential
240 to modulate activity rhythms as populations face new environmental and/or social contexts. Our
241 findings suggest the evolution of highly disparate locomotor patterns, diurnality and nocturnality,
242 are influenced by environmental/social context. These findings raise the possibility that the
243 evolution of complex social behaviors co-evolve with rest-activity patterns to contribute to the
244 regulation of circadian behavior.

245

246

247 **Biological mechanisms of circadian regulation**

248 The hypothalamus is the primary regulator of circadian behavior and outputs from the
249 suprachiasmatic nucleus are thought to convey diurnal or nocturnal behavior^{41,42}. In zebrafish, the
250 SCN contains core-transcriptional clock genes common in mammals suggesting a conserved
251 function in the regulation of locomotor patterns, although the presence of many light-responsive
252 tissues in teleosts suggests clock regulation may be more complicated than in mammals⁴¹. In
253 *Drosophila* through mammals social interactions contribute to the regulation of sleep and
254 circadian behavior. In mammals, social interactions impact clock entrainment. For example, social
255 defeat decreases the amplitude of the core-clock gene *Per2* in the periphery, as well as sleep
256 homeostasis^{43,44}. There is reduced complexity of social interactions in young juvenile fish that
257 displayed robust rhythms^{35,45}. However, we find that activity patterns are absent in individually
258 housed adult fish. These findings raise the possibility that unlike mammals, stress that impairs
259 circadian function comes from solitary housing, rather than social stress. Indeed, in *Drosophila*
260 solitary housing is associated with sleep dysregulation and disruption of circadian gene
261 expression^{46,47}. Understanding both the SCN outputs, and how they are modulated by social
262 context provides a mechanism for understanding how context-dependent regulation of activity
263 patterns evolved. Our findings raise the possibility that these outputs are modulated by social
264 circuits, or other context-specific regulators of behavior.

265

266

267 **Adaptive significance**

268 Development of diversity in behavioral rhythms in cichlids may be a type of habitat partitioning,
269 with cichlid species altering the timing of their behaviors to take advantage of reduced competition
270 for resources such as food and territory²⁴. The presence of larger nocturnal predators in Lake
271 Malawi, such as the Cornish Jack *Mormyrops anguilloides*, presents a constraint to this
272 adaptation, requiring nocturnal cichlids to develop strategies for avoiding predation at night²³. It
273 has been previously reported that *A. stuartgranti* possesses widened lateral line canals which
274 enable them to detect prey in the dark, but this same adaptation may also enable detection and
275 evasion of predators during the dark period⁴⁸. It is interesting that both nocturnal species are also
276 crepuscular, and both are predatory. There is evidence that predator-prey interactions are highest
277 during the twilight hours, and crepuscularity may be an adaptation in predators to facilitate access
278 to both day-active and night-active prey^{49,51}. Also notable is that neither species recovered
279 rhythmicity when placed in a group context. This may reflect differences in the level of social
280 interaction in these fish in the wild, such that it is not group context, but rather a yet unidentified

281 factor that is required for adult activity rhythms. It is also possible that rest-activity rhythms are
282 truly lost in these species over ontogeny. In short, there is still much to learn about the regulation
283 of activity patterns in this system, and testing hypotheses about the adaptive significance of these
284 behaviors will require the application of phylogenetic methods on a much larger sampling of taxa.

285

286

287 **Challenges and Future Directions**

288 It has been found that changes in not just light quantity, but also composition (color), occur during
289 twilight, and that the circadian clock machinery is responsive to this change^{50,52}. Because our
290 study used constant levels of white light throughout the day, we were unable to evaluate the
291 effects of changes in light quality on behavioral rhythms; future studies may address this question
292 by using simulated dusk/dawn transitions.

293

294 The duration of circadian experiments provide a significant challenge for analyzing data across
295 multiple species. Here, we used multiple approaches to quantify locomotor activity in group-
296 housed fish. First, we used Ethovision to track total activity. While this system is capable of
297 tracking individual fish in a shared arena, the tracks regularly cross-over preventing
298 measurements of activity in individuals over a 24 hour period. To identify the activity of individuals
299 we visually quantified behavior using BORIS. While this approach allows for reliable behavioral
300 measurements in individual fish it lacks precise quantification. Given the time-consuming nature
301 of the analysis, we only analyzed a single experiment of six fish. Recent development in
302 automated tracking including DeepLabCut and IDTracker may provide applications for long-term
303 automated tracking^{53,54}. These systems have been effectively applied to measure social behaviors
304 and activity in populations of animals^{53,55}. Further, we have tracked large groups of *A. mexicanus*
305 to examine social interactions³². While these applications have yet to be applied to long-term
306 analysis of rest-activity patterns the rapid improvements in processing speed and accuracy are
307 likely to allow for analysis of sleep and circadian regulation of activity under social contexts.

308

309 It is widely recognized that rest-activity patterns are regulated by many life-history and
310 environmental traits^{56,57}. The growing use of animal models in rest-activity regulation has provided
311 unprecedented insight into the genetic, neural and evolutionary processes that govern these
312 behaviors. However, the vast majority of studies still test animals under individually housed
313 conditions because it provides a simpler method of data acquisition and the removal of variables
314 that may impact behavior. The results of this study suggest that developmental stage and

315 environmental conditions can have profound effects on behavioral regulation, and highlight the
316 need for a more thorough investigation of social influences and other factors that may play a role
317 in regulation of behavior.

318

319

320 **Materials and Methods**

321 **Fish husbandry**^{37,38}

322 Cichlids used for experiments were reared following standard protocols approved by the Texas
323 A&M University Institutional Animal Care and Use Committee. Cichlids were housed in the Keene
324 fish facilities at Texas A&M University at a water temperature of 28.5°C, on a 14 h:10h light:dark
325 cycle. Adult cichlids were fed TetraMin tropical flakes (TetraMin) twice a day. Juvenile cichlids
326 were fed live *Artemia* brine shrimp twice daily.

327

328 **Fish breeding**

329 Breeding was facilitated by the inclusion of clay pots in the cichlid's home tanks, which provided
330 arenas for mating behaviors. Breeding occurred spontaneously, and females were periodically
331 visually inspected for an enlarged buccal cavity, evidence of fertilized eggs. For experiments
332 testing the behavior of naturally emerged juveniles, mouthbrooding females were isolated and
333 their tank was checked daily for the emergence of juveniles. Upon emergence, juveniles were
334 transferred to the experimental setup immediately. For all other experiments, mouthbrooding
335 females were allowed to carry their embryos until the hatching stage (approximately 3-5 days). A
336 waiting period prior to extraction increased survival rates of the offspring in our hands.

337

338 To extract fertilized eggs, the mouthbrooding female was transferred to a holding tank and briefly
339 restrained by hand, while the mouth was gently opened with the pad of the thumb. The buccal
340 cavity was gently stroked to facilitate egg removal. Following extraction, the female was returned
341 to her home tank, and the eggs were transferred to a 1000mL capacity Erlenmeyer flask (VWR,
342 10545-842) with an air stone bubbler to maintain oxygen levels. Eggs were left to develop until
343 free-swimming, and the yolk mostly depleted (approximately 3-4 weeks), at which point they were
344 transferred to the experimental setup immediately.

345

346 **Behavior measurements**

347 For experiments testing locomotor activity in juvenile fish, juveniles were transferred to individual
348 wells of 6-well culture plates (Falcon, 353046), and placed on a light box constructed of white 1/8"

349 high-density polyethylene plastic (TAP Plastics), which allowed for even diffusion of light to
350 facilitate automated tracking. Light boxes were lit from below with infrared light, using 850 nm
351 LED strips (Environmental Lights). Fish were acclimated to the testing chambers overnight;
352 behavioral recording began the following day at ZT1, and ran for 24 hours. Juveniles were filmed
353 from above, at 15 frames per second with a USB camera (LifeCam Studio 1080p, Microsoft),
354 modified to remove the IR-blocking filter, and with an IR-pass filter (Edmund optics, 43-948) added
355 to ensure consistent lighting during the light and dark periods.

356
357 For experiments testing locomotor activity in isolated adult fish, adults were transferred to 2.5
358 gallon tanks (Carolina Biological Supply, 671226), fitted on the floor and walls with custom-cut
359 white corrugated plastic (3mm thick), allowing for even diffusion of light to facilitate automated
360 tracking. Tanks were lit from behind with infrared light. Fish were acclimated to the testing
361 chambers overnight; behavioral recording began the following day at ZT1, and ran for 24 hours.
362 Adults were filmed from the side, with the same recording equipment described above.

363
364 For experiments testing locomotor activity in group-housed adult fish, adults were transferred to
365 10 gallon tanks (Carolina Biological Supply, 671230), fitted on the floor and walls with custom-cut
366 white corrugated plastic, and lit from behind with infrared light. Fish were acclimated to the testing
367 chambers overnight; behavioral recording began the following day at ZT1, and ran for 24 hours.
368 Each group consisted of 2 male and 3-4 female conspecifics.

369
370 **Automated behavioral tracking**
371 Acquired videos were processed in Ethovision XT 15 (Noldus), and positional data over the 24
372 hour period was extracted and analyzed using a custom-made Python script (v 3.11.3) to calculate
373 locomotor activity on an hourly basis. Analysis of rest patterns was carried out as previously
374 described²⁴, with a threshold of 4 cm/s for adults, and 12 cm/s for juveniles. Bouts of inactivity
375 greater than 60 seconds were considered rest.

376
377 For group housed experiments, locomotor activity was calculated using Ethovision's Social
378 Interaction Module (Noldus). Due to limitations of the software, the identities of individual fish
379 could not be maintained over the 24 hour period, so the average locomotion within each group
380 was used for subsequent analysis.

381
382 **Manual behavior scoring**

383 To assess individual behavioral rhythms within group housed cichlids, a single 24-hour video was
384 manually scored in BORIS, an interactive behavior logging software³⁶. Active swimming behavior
385 was logged as a state event (on/off), according to the following criteria: “Focal fish can be seen
386 actively swimming with appendicular movements even though the pace may change throughout
387 the duration of one bout of movement”. A full 24 hours was logged for each fish (N=2 males, N=4
388 females) in a single video. The identity of the dominant male was determined by his larger size
389 and nuptial coloration pattern. The females were also identified by size, with the largest female
390 designated “Female 1” and the smallest “Female 4”. A single scorer performed all manual activity
391 logging, to avoid inter-individual differences in evaluation of criteria. Data for each fish was
392 exported in the “Aggregated Events” format, and then analyzed using a custom-made Python
393 script (v3.11.3) to extract hourly measurements of locomotion. Rest bouts were calculated from
394 the aggregated movement data, with any period of inactivity greater than 60 seconds considered
395 a rest bout.

396

397 **Statistical Analysis**

398 To identify differences between multiple conditions, such as activity in the light versus dark, or
399 between juvenile and adult behavior, a two-way ANOVA was carried out, and followed by Šidák's
400 multiple comparisons post hoc test. All statistical testing was carried out using InStat software
401 (GraphPad Prism 9.5).

402

403 Diurnality was calculated as described previously, as an “activity change ratio”, with $A_R = \frac{D-N}{D+N}$,
404 where D and N are average hourly activity during the day and night. Crepuscularity was calculated
405 as $C_R = \frac{C}{NC} - 1$, where C is equal to the average activity in the hours following light transitions
406 (i.e. dawn and dusk), and NC is equal to the average activity across the rest of the day. In this
407 formulation, 0 represents no change in activity following a light transition, and 1 represents a
408 100% increase in activity levels²⁴.

409

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574 **Figure Legends**

575

576 **Figure 1. Variation in cichlid activity levels is conserved throughout development. A.**

577 Images of early juvenile (left) and adult (right) cichlid species used in the present study.

578 Juveniles were tested and photographed between 3-4 weeks post-fertilization, shortly after the

579 depletion of the yolk sac and at the earliest free-swimming stage. Adults were tested and

580 photographed at full maturity, after the development of nuptial colors. White Scale Bar

581 (Juveniles) = 1 mm. Black Scale Bar (Adults) = 1 cm. **B.** Total locomotion of juvenile cichlids

582 over a 24 hour period. There is significant variation in total locomotion in juvenile cichlids

583 (ANOVA: $F_{5, 190} = 15.99, p < 0.0001$). **C.** Total locomotion of adult cichlids over a 24 hour period.

584 There is significant variation in total locomotion in adult cichlids (ANOVA: $F_{5, 67} = 6.42,$

585 $p < 0.0001$) **D.** Correlation between 24-hour locomotion in juveniles and adults. There is a

586 significant correlation between juvenile and adult locomotion ($r^2 = 0.70, p = 0.0388$)

587 **Figure 2. Ontogeny of behavioral rhythms in locomotion. A.** Locomotion profiles of juvenile

588 cichlids over 24 hours. Shaded area indicates dark period. **B.** Locomotion profiles of adult

589 cichlids over 24 hours. Shaded area indicates dark period. **C.** Average hourly locomotion of

590 juvenile cichlids during the day and night. Juvenile cichlids exhibit diurnal (*A. calliptera*, *L.*

591 *caeruleus*, *M. heterochromis*, *T. kumwera*) or nocturnal (*A. stuartgranti*, *N. venustus*) activity

592 patterns (two-way ANOVA: $F_{5, 190} = 36.54, p < 0.0001$). **D.** Average hourly locomotion of juvenile

593 cichlids during the day and night. Adult cichlids lack behavioral rhythms when tested in isolation.

594 **E.** Strength of behavioral rhythms in juvenile and adult cichlids. 1 indicates total diurnality, -1

595 indicates total nocturnality. Behavioral rhythms are present in juveniles but not adults (two-way

596 ANOVA: $F_{5, 280} = 16.20, p < 0.0001$).

597 **Figure 3. Ontogeny of behavioral rhythms in rest. A.** Rest profiles in juvenile cichlids over 24

598 hours. Shaded area indicates dark period. **B.** Rest profiles in adult cichlids over 24 hours.

599 Shaded area indicates dark period. **C.** Total rest amounts over 24 hours in juvenile cichlids.

600 There is significant variation in total rest amount in juvenile cichlids. (ANOVA: $F_{5, 190} = 42.4,$

601 $p < 0.0001$). **D.** Total rest amounts over 24 hours in adult cichlids. There is significant variation in

602 total rest amount in adult cichlids (ANOVA: $F_{5, 67} = 8.792, p < 0.0001$). **E.** Correlation between

603 juvenile and adult rest amounts. There is significant correlation between juvenile and adult rest

604 ($r^2 = 0.82, p < 0.0128$). **F.** Average hourly rest amount of juvenile cichlids during the day and

605 night. Juvenile cichlids exhibit diurnal (*A. calliptera*, *L. caeruleus*, *T. kumwera*, *N. venustus*) or

606 nocturnal (*A. stuartgranti*) rest patterns (two-way ANOVA: $F_{5, 358} = 10.95$, $p < 0.0001$). **G.** Average
607 hourly rest amount of adult cichlids lack behavioral rhythms in rest amount.

608 **Figure 4. Social context restores rhythms in diurnal cichlids.** **A.** Schematic of test setup.
609 Adult cichlids are transferred to a 10-gallon testing aquarium, acclimated overnight, and then
610 recorded for 24 hours. **B.** Average locomotion of cichlid groups over 24 hours. Shaded area
611 indicates dark period. **C.** Average hourly locomotion of group housed cichlids, during the day
612 and night. **C.** Average hourly locomotion of group housed cichlids during the day and night,
613 grouped into diurnal and nocturnal species. Group housing restores rhythmic behavior in
614 diurnal, but not nocturnal species (two-way ANOVA: $F_{1,6} = 11.23$ $p = 0.0154$). Colors of data
615 points indicate species.

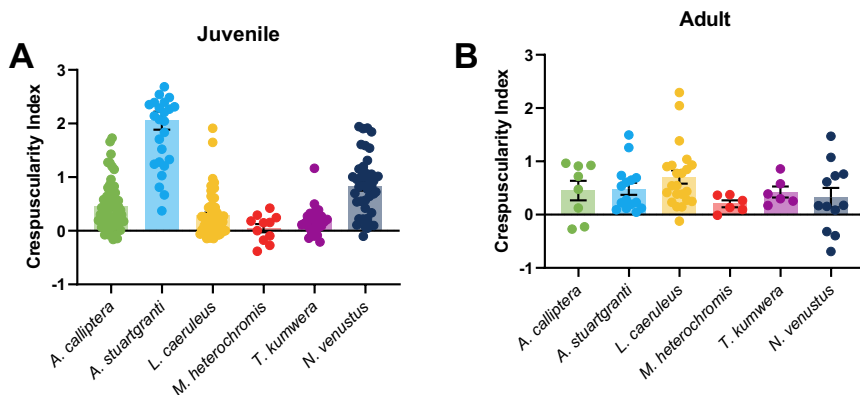
616 **Figure 5. Individual variation in behavioral rhythms in a social context.** **A.** Ethogram of
617 active swimming behavior of *L. caeruleus* over 24 hours, in a social context. Colored regions
618 indicate periods of active swimming. Shaded area indicates dark period. **B.** Profile of locomotion
619 in group housed *L. caeruleus*, quantified as minutes of active swimming per hour. Shaded area
620 indicates dark period. **C.** Average hourly locomotor activity of group housed *L. caeruleus* during
621 the day and night. Group housed *L. caeruleus* are significantly more active during the day
622 (Paired t-test: $t_5 = 6.413$, $p = 0.0014$). **D.** Rest profile of group housed *L. caeruleus* over 24
623 hours. Shaded area indicates dark period. **E.** Average hourly rest of group housed *L. caeruleus*
624 during the day and night. Group housed *L. caeruleus* consolidate rest during the dark period
625 (Paired t-test: $t_5 = 5.198$, $p = 0.0035$).

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629 Supplemental Figures



Supplemental Figure 1. Ontogeny of crepuscular behavior. **A.** Crepuscularity index of juvenile cichlids. There is significant variation in degree of crepuscular behavior among juvenile cichlids (ANOVA: $F_{5, 190} = 55.04$, $p < 0.0001$). **B.** Crepuscularity index of adult cichlids. There is no significant variation in degree of crepuscular behavior in adult cichlids.